THE ROLE OF Ca^{++} IN PEPSINOGEN RELEASE BY ISOLATED RABBIT GASTRIC MUCOSA

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Signature of Author

April 2, 1979

Date

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ABSTRACT

The role of Ca^{++} in the secretion of pepsinogen was studied *in vitro* in rabbit gastric mucosa. While extracellular Ca^{++} was not shown to be essential for ACh-induced secretion to occur, maximal secretion did not occur if Ca^{++} was omitted from the medium. Also, increasing the extracellular Ca^{++} concentration did not enhance secretion.

Three drugs which reportedly increase the intracellular Ca^{++} concentration were studied for their ability to stimulate secretion. X537A (a non-specific Ca^{++} ionophore) and veratridine (a depolarizing agent) both induced secretion, although submaximal. No secretion was demonstrated with A23187, a more specific Ca^{++} ionophore.

These results support the concept that Ca^{++} is involved in pepsinogen secretion, although the role of Ca^{++} as mediator in this stimulus-secretion process has not been substantiated. Intracellular Ca^{++} may actually have a more important role than extracellular Ca^{++}.

Also, the ability of X537A and veratridine to induce secretion may be due to Na^{+} influx and depolarization with resulting increased membrane permeability to calcium ions. A23187, which does not transport Na^{+}, was unable to stimulate pepsinogen secretion.

Finally, strontium was studied for its ability to substitute for Ca^{++} in the secretory process. Sr^{++} was able to support ACh-induced pepsinogen secretion but was less effective than Ca^{++}.

products[12-14]. Morphologically, therefore, secretion was viewed as a process involving extrusion of granules containing secretory product. Biochemically, the factors which initiate and affect this process remained to be elucidated.

It was Douglas who set forth the hypothesis that the secretogogue ACh increased cell membrane permeability, allowing Ca^{++} influx, and that Ca^{++} was a general mediator of secretion. This "stimulus-secretion" process is comparable to the excitation-contraction coupling in muscle, for which Ca^{++} has been shown to have a mediator role. Douglas' proposal was based on experiments with chromaffin cells of the adrenal medulla. This system offered several advantages:

1. The medulla is embryologically related to the neuron, where previous work with Ca^{++} had been done.

2. The medulla has large stores of pre-formed catecholamine stored in granules[15]. Therefore, release rather than synthesis could be observed.

A direct relationship was found between the extracellular Ca^{++} concentration and the amount of catecholamine release stimulated by ACh. In a Ca^{++}- free environment release was either greatly inhibited or completely abolished; addition of Ca^{++} restored the secretory response[17]. Under other conditions in which membrane permeability had been increased (such as after a time of Ca^{++} deprivation or addition of excess K^{+}) Ca^{++} itself was able to stimulate secretion. Also, it was found that ACh caused an eightfold increase in Ca^{++} uptake by the medulla. This increased uptake had been documented previously in various muscles[18-20].

I. INTRODUCTION

A. Ca^{++} and the secretory process

Since the initial studies of W. W. Douglas, evidence has accumulated to firmly support the role of Ca^{++} in the exocytosis of packaged secretions in a variety of systems. Ca^{++} has been shown to mediate secretion of norepinephrine from adrenergic nerves[1]; insulin from beta-pancreatic cells[2]; histamine and serotonin from mast cells and platelets[3]; trophic hormones from the adenohypophysis[4]; HCl and gastrin from the stomach[5]; GABA and glutamic acid from brain synaptosomes[6].

The importance of Ca^{++} in the secretory process was studied at the neuromuscular junction as early as 1894 when Locke discovered that addition of Ca^{++} to frog sartorius muscle bathed in a Ca^{++}- free saline solution restored the ability of the muscle to respond to nerve stimulation[7]. In 1940 Harvey and MacIntosh[8] demonstrated inhibition of acetylcholine (ACh) release from pre-ganglionic nerve endings (upon nerve stimulation or addition of K^{+}) when Ca^{++} was omitted from the perfusing medium. Likewise Katz[9], using microelectrode techniques, showed that Ca^{++} has a direct role in the release of quanta of ACh at the frog neuromuscular junction.

Electron microscopy in the late 1950's, early 1960's indicated that some cells used 'reverse micropinocytosis" or exocytosis to release secretory material[10]. This was well-demonstrated in the pancreatic acinar cell by Palade in 1958[11] and exocytosis soon became established as a general mechanism in the release of secretory

The Ca^{++} requirement was then shown to be specific, i.e. neither Na^{+} nor K^{+} was required in the perfusion media to elicit release. Also, while K^{+} increases the membrane permeability, in the absence of Ca^{++} secretion was not observed. This inability of increased permeability alone to cause secretion is further supported by the fact that the adrenal medulla is stimulated by diverse chemicals but only in the presence of Ca^{++}.

Douglas next studied the neurohypophysis which has a common developmental origin with chromaffin cells and has many morphological and electrophysiological characteristics similar to neurons. In this system, stimulation (either electrical or with K^{+}) led to Ca^{++} influx and secretion; the response was Ca^{++}- dependent[21]. Thus, in the hypothalamo-neurohypophyseal system, generation and propagation of the action potential causes depolarization of the nerve terminal with entry of Ca^{++} and subsequently exocytosis[22].

The role of calcium in secretion was demonstrated not only in neural tissue but also in exocrine glands (i.e. submaxillary salivary gland)[23] and in the mast cell[24]. It therefore seemed reasonable when Douglas, in 1968, proposed that Ca^{++} is a general mediator of secretion: various substances or stimuli alter membrane permeability leading to Ca^{++} influx which in turn leads to secretion. Such a mechanism was believed to operate in neurons, neurosecretory fibers, endocrine and exocrine cells, and other secretory cells i.e. mast cells. The stimuli included action potentials, chemical transmitters, hormones, autacoids, bacterial toxins, and antigens[25]. Thus, while secretory cells may vary extensively in embryology, morphology,

among different systems. For example, cAMP stimulates secretion in fly salivary gland but inhibits secretion from mast cells. Furthermore, cAMP appears to have no effect on adrenal medulla[30].

Since Ca^{++} deprivation does not decrease cAMP production one possibility is that cAMP activates secretion by redistributing Ca^{++}. Another hypothesis is that cAMP activates cellular protein. Rasmussen[29] has proposed that cAMP activates a protein kinase which in turn phosphorylates microtubular protein. This converts the cytoskeleton from a Ca^{++}- insensitive to a Ca^{++}- sensitive state. Ca^{++} is then able to activate the phosphorylated protein, leading to secretion.

Although cAMP has been studied more than the other cyclic nucleotides, there is also evidence that cGMP and Ca^{++} are interrelated in the secretory process. Prostaglandins have also been implicated; they induce secretion from exocrine and endocrine pancreas[31], thyroid[32], and adrenal cortex[33]. Thus, while Ca^{++} is thought to be a mediator in the secretory process, the role of cyclic nucleotides and other substances remains to be clarified.

B. Role of other divalent cations

Another area of investigation has involved the ability of divalent cations other than Ca^{++} to evoke the stimulus-secretion response. Douglas & Rubin[34] found that Sr^{++} was an equally effective substitute. When cat adrenal medulla was perfused with Ca^{++}- free Locke's solution, addition of Sr^{++} restored the secretory responses to ACh or K^{+}. Sr^{++} appeared to directly activate the secretory

electrical properties, and secretory product, secretion was thought to involve a basic process similar in all these cells.

The belief that secretogogues act on the plasma membrane, leading to increased permeability, Ca^{++} entry, and thus secretion has been strongly supported by work with the adrenal medulla and electrically excitable tissue. However, in other systems it is believed that the secretogogues effect a redistribution of cell Ca^{++} rather than Ca^{++} influx. ACTH and TSH, for example, not only act on the plasma membrane but also cause Ca^{++} redistribution. Insulin secretion may also involve a shift of Ca^{++} to a less mobile pool, thereby increasing the amount of cellular Ca^{++} [27]. Whether the pool of Ca^{++} is extracellular or intracellular, it is generally held that Ca^{++} is involved in the stimulus-secretion process. It has <u>not</u> been proven beyond a reasonable doubt that Ca^{++} is a direct link between the stimulus and exocytosis, as set forth in the original hypothesis by Douglas.

To complicate matters further, there are other substances (such as the cyclic nucleotides) which may have an important role in the stimulus-secretion process. Ever since cAMP was found to mediate the glycogenolytic effect of epinephrine on liver homogenates in 1957[28] the actions of many hormones have been found to involve cAMP as mediator. Recently the role of cyclic nucleotides in secretion has received much attention. Studies of various systems have led to the hypothesis that cyclic nucleotides either directly or indirectly regulate intracellular Ca^{++} metabolism, and also that Ca^{++} affects cyclic nucleotide metabolism[29]. The relationship, however, varies

mechanism rather than mobilize intracellular Ca^{++}.

At motor nerve endings, Sr^{++} was able to activate neurotransmitter release but was quantitatively much less effective than Ca^{++} (2.0 mM Sr^{++} produced an effect equivalent to 0.3 mM Ca^{++}). This difference in potency seems related to an ability to directly activate the secretory mechanism rather than to differing affinities for hypothetical critical sites[35].

In contrast, Sr^{++} was found to be more effective than Ca^{++} in producing histamine release from mast cells. By kinetic studies it was concluded that Sr^{++} and Ca^{+++} act on similar sites but that Sr^{++} had greater "efficacy" at these sites[36].

Like Sr^{++} and Ca^{++}, Ba^{++} is able to stimulate secretion from nerves, adrenal medulla, mast cells, etc. However, an increase in cell permeability provided by ACh, excess K^{+}, or Ca^{++} deprivation is not required; Ba^{++} acts by depolarizing the cell. This stimulant action is blocked by Mg^{++} and inhibited by Ca^{++}. It is possible that Ba^{++} increases cell permeability by displacing Ca^{++} from the plasma membrane[37].

The fact that Sr^{++} and Ba^{++} are divalent cations does not explain their ability to substitute for Ca^{++}. Mg^{++}, Ni^{++}, Co^{++}, Zn^{++}, and Mn^{++} are all ineffective in the secretory process. One possibility is that these cations are unable to reach the critical sites involved. However, these cations have hydrated ion radii smaller than Ba^{++}, Sr^{++} or Ca^{++} [38]. Also, Mg^{++} has been shown to inhibit the actions of Ca^{++} in many systems[39]. Perhaps, therefore, the ineffective divalent cations are unable to activate the

secretory apparatus even when presented to it[34].

C. Pepsinogen secretion

It is apparent from the foregoing discussion that the secretory process is being extensively investigated in a variety of systems (i.e. mast cells, pancreas, salivary glands, neurons, adrenal medulla, etc.). Surprisingly, however, the area of gastric secretion has received little attention. It was the purpose of this thesis, therefore, to study the secretion of pepsinogen (using rabbit gastric mucosa as the model) and to perhaps determine whether Ca^{++} is indeed the mediator between stimulus and secretion.

Pepsinogen is the inactive precursor of the proteolytic enzyme pepsin and is found in the peptic (chief) cell, blood, and other body fluids. It is a protein (molecular weight 42,500) first crystallized from gastric mucosa by Herriott[40], and is composed of pepsin and peptide fragments. One of these fragments, the inhibitor (M.W. 3,100), is the terminal part of the pepsinogen molecule. Below pH 5.4 the pepsin-inhibitor complex dissociates; at pH 3.5-4.0 the inhibitor is digested by pepsin.

The peptic cell was first differentiated from the parietal cell in 1870 by Rollitt and independently by Heidenhain, and the function was elucidated in the next decade by Langley[41]. Absent from the antrum, the peptic cells are present in the gastric body and fundus; in mammals they are located in the lower two-thirds of the gastric tubules with the parietal cells occupying the upper one-third.

Similar to other secretory cells (i.e. pancreas, salivary gland)

the peptic cell originates from the endoderm and undergoes distinct histologic changes in relation to secretory activity. Its general morphology has been studied by light and electron microscopy[42-45] and its ultrastructure includes zymogen granules and a tubular arrangement of endoplasmic reticulum (ER). It is believed that pepsinogen is synthesized in the polysome-mRNA system on the ER, is transferred into the tubular system of the ER, and is then transported to the Golgi body where encapsulation occurs. The granules are first argyrophilic but as they reach the secretory surface they become argyrophobic and stainable by crystal violet. The ability of the pepsinogen granule to take up crystal violet is most likely due to the presence of an unidentified ground substance and not to pepsinogen itself. The peptic cell produces not only pepsinogen but also contains closely-related proteolytic enzymes[46] as well as intrinsic factor. (In some species, including man and rabbit, intrinsic factor is made by the parietal cell.)

Upon stimulation of the peptic cell there is a rapid discharge of stored granules resulting in an initially high concentration of pepsin[47,48]. It has been shown that after secretion there occurs a marked increase in cytolasmic RNA which is used in the synthesis of new protein. Thus it is believed that the loss of granules, by a positive feedback mechanism, stimulates further pepsinogen synthesis. If the original stimulus to secretion is short-lived, synthesis begins to replenish pepsinogen stores over several hours. However, if the stimulus is continuous synthesis reaches maximum rate in 45-60 minutes until it equals the rate of secretion.

The role of Ca^{++} on gastric secretion was investigated as early as 1941 by Gray and Adkinson[49]. On the basis of Ca^{++} deprivation studies, they suggested that Ca^{++} affected the secretory process and electrical properties of frog gastric mucosa. While quantitative *in vitro* studies relating Ca^{++} concentration to gastric secretion have been lacking, it is well-known in man that Ca^{++} administration increases gastric secretion. Also, there is a high incidence of peptic ulcers in hyperparathyroidism, a syndrome accompanied by hypercalcemia. Ca^{++} produces an increase not only in acid production but also in pepsin and gastrin secretion[50,51].

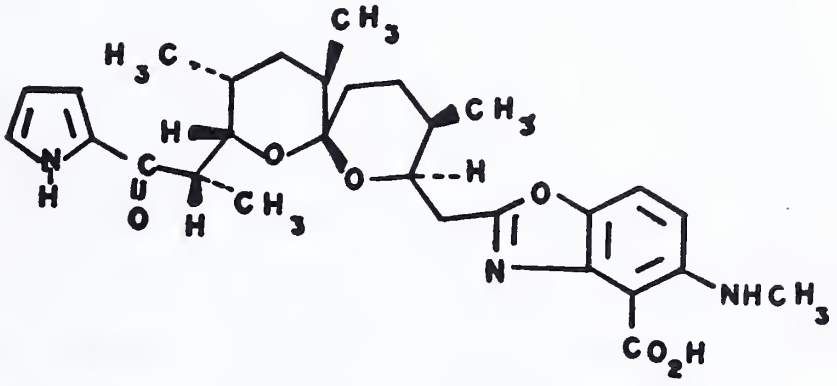
A major drawback to these *in vivo* studies is that secretion is affected by both neural and hormonal influences and the site of Ca^{++} action cannot be determined. It is true that, *in vivo*, hypermagnesemia can inhibit the action of Ca^{++} on gastric secretion and this has been observed *in vitro* in many other systems (such as the adrenal medulla). However, the Ca^{++} effect can also be blocked *in vivo* by denervation or atropine[52] and therefore the possibility exists that the Ca^{++} effect is mediated by ACh from the vagus nerve. On the other hand, cholinergic stimuli, gastrin, histamine, caffeine, reserpine, and hyperventilation all stimulate pepsinogen secretion in man, but only the cholinergic stimuli and gastrin are sensitive to atropine. It appears highly unlikely that any one of these stimuli is the common mediator for pepsinogen secretion.

In order to eliminate as many variable as possible, therefore, *in vitro* rabbit gastric mucosa was used in this study of pepsinogen secretion.

D. Ca^{++} ionophores

Another important part of this study involved the use of Ca^{++} ionophores. An ionophore is "a compound which facilitates the transport of an ion through a natural or artificial lipid membrane from one aqueous medium to another."[30] Two such ionophores are X537A and A23187, both monocarboxylic acids. A23187 has been more extensively used than X537A in secretion studies since it has been shown to be more specifc for Ca^{++} (see figure 1). In essence, A23187 catalyzes an electroneutral exchange of protons for divalent cations across membranes of cells and organelles, i.e. mitochondria[54]. It is believed that the cation crosses the membrane complexed to two molecules of A23187 which are in the carboxylate anion form and which transport two equivalents of H^{+} back across (after first releasing the cation). Divalent rather than monovalent cations are transported but this selectivity is not absolute. Transport of K^{+} by A23187 in rat mitochondria was demonstrated, and formation constants for the A23187-cation complex indicate that transport of Li^{+} and Na^{+} would also be possible with appropriate conditions[54]. Furthermore, both X537A and A23187 form complexes not only with Ca^{++} but also with other divalent cations (i.e. Ba^{++}, Sr^{++}) and transport them across membranes[55]. In fact, X537A has a higher affinity for complexing with Ba^{++} and Sr^{++} than with Ca^{++}. In a preparation of guinea pig vasa deferentia, in the presence of Ba^{++}, X537A induced a cholinergic transmitter release four times that produced in the presence of Ca^{++}.

A23187



Produced by fermentation of Streptomyces chartreusis

Molecular Formula: $C_{29}H_{37}N_3O_6$

Molecular Weight: 523

FIGURE 1. STRUCTURE OF A23187

The exact mechanisms by which A23187 act are not known although various theories have been proposed. One hypothesis has been that A23187 increases plasma membrane permeability to Ca^{++}, leading to influx of extracellular Ca^{++}; the resulting increased cytoplasmic Ca^{++} concentration then is able to induce exocytosis. While it is true that A23187 increases Ca^{++} fluxes across plasma membrane, it does not necessarily follow that A23187 acts only at the plasma membrane. Compounds which are incorporated into membranes such as mitochondrial uncouplers (DNP) and fluorescent probes (chlorotetracycline) have been localized at intracellular organelles as well as the plasma membrane. The ability of A23187 to increase Ca^{++} membrane permeability has been demonstrated in isolated mitochondria[59] and isolated sarcoplasmic reticulum[56] as well as in intact cells[57]. Also, even in the absence of extracellular Ca^{++}, A23187 can stimulate secretion or contraction in some cells[58]. Therefore, while the data support the idea that Ca^{++} ionophores increase free Ca^{++} concentration in the cell cytoplasm, the source of this Ca^{++} may not be only extracellular.

It is apparent that in some systems the required Ca^{++} is not solely extracellular. Secretion in platelets involves the intracellular Ca^{++} pool, while in the fly salivary gland it seems that Ca^{++} is made available from both the intra- and extra-cellular compartments[61]. The theory that stimulus-secretion coupling involves a redistribution of intracellular Ca^{++} is supported by Chandler et al[60]. It was demonstrated with the use of a fluorescent probe (CTC) that Ca^{++} is released from intracellular membranes during bethanechol stimulation of

pancreatic acinar cells. It seems that secretion is induced by a rise in the free Ca^{++} concentration in the cytosol and that cell membrane permeability to Ca^{++}, mitochondrial Ca^{++} pumps, and membrane Ca^{++} pumps may all be involved.

One disadvantage to using ionophores rather than physiologic secretogogues is the possibility that the pool of Ca^{++} involved may be different. Also, it has been shown that the physiologic first messenger and A23187 have different effects on Ca^{++} entry[61]. The rate of Ca^{++} influx is directly related to the concentration of physiologic messenger, up to a maximum. At this point an increase in messenger does not elicit a further increase in Ca^{++} entry. With A23187, however, no maximum is reached and increasing concentrations of A23187 will cause increasing rates of Ca^{++} influx.

Another difficulty has been that Ca^{++} ionophores reportedly decrease the total cell Ca^{++} concentration in various systems. However, this finding is not incompatible with the idea that the ionophores increase the intracellular Ca^{++} concentration. Experiments with red blood cells (RBC) have shown that cellular Ca^{++} uptake and total cell Ca^{++} concentration are greatly increased in the presence of A23187, but RBC are unique in that they lack mitochondria. In other models, Ca^{++} influx into the cell and Ca^{++} release from mitochondria both occur and the net effect may be a loss of Ca^{++}. For example, A23187 was found to have separate effects at the plasma and mitochondrial membranes of bovine epididymal spermatozoa. Low concentrations of the ionophore were associated with efflux of Ca^{++} as well as stimulation of respiration, while high concentrations induced

influx. It was concluded that the ionophore, in low concentrations, rapidly localized in the mitochondrial membrane and caused release of Ca^{++} [57]. This effect has also been demonstrated in isolated rat liver mitochondria[59,64]. Therefore, ionophores may not always act solely on the plasma membrane.

Also, while X537A and A23187 are both Ca^{++} ionophores and induce secretion their mechanism of action probably differs. For instance, their effectiveness as secretogogues is not directly proportional to their ability to transfer Ca^{++} ions. X537A is the less effective Ca^{++} ionophore yet is much more potent in stimulating catecholamine release from the cat adrenal[65]. Whereas X537A induced vasopressin secretion from rat neurohypophysis, A23187 had no effect[66]. One explanation is based on the fact that X537A can transport monovalent cations while A23187 cannot. Also, depolarization has been shown to be an adequate stimulus for secretion[65]. Therefore, it has been proposed that X537A transports monovalent cations across cell membranes, causing depolarization and increased membrane permeability which in turn leads to influx of Ca^{++} and secretion. This hypothesis is supported by various systems in which excess Mg^{++} (found to interfere with the depolarization-induced Ca^{++} influx) inhibits release.

E. Veratridine

Another compound useful in studying stimulus-secretion is the neurotoxin veratridine, a steroidal alkaloid from plants of the family Liliaceae (suborder Melanthaceae). Veratridine causes depolarization

and repetitive firing of nerves. Because this action is blocked by tetrodotoxin (a specific inhibitor of the action potential Na^+ current) and since neuroblastoma clones lacking the depolarizing phase of the action potential spike do not respond to veratridine, it is believed that veratridine activates the action potential Na^+ ionophore. The idea that veratridine increases membrane permeability to Na^+ which leads to depolarization and then increased Ca^{++} permeability is supported by experiments with rat brain synaptosomes[67,68], the squid giant synapse[69], muscle and neuroblastoma cells[66]. Like the secretion stimulated by excess K^+ or the ionophore A23187, release induced by veratridine has been shown to be Ca^{++}- dependent[68].

II. Methods and Materials

A. Preparation of Gastric Mucosa

Male fasted New Zealand white rabbits weighing 2-4 kgs. were anesthetized with ether and their stomachs were removed. The lesser curvature was incised; the stomach was then everted and immediately immersed in cold 0.9% saline. The mucosa was gently cleaned with cotton gauze. Next, the antrum was discarded and a 3 x 5 cm. portion of fundus was held fairly taut with 4 hemostats. The tissue was washed with gauze and cold control media until the rugae were no longer visible. A #3 scalpel handle was used to strip the mucosa away from the underlying tissue, and with a #1 cork borer tissue biopsies (5-7 mg) were obtained from the gastric mucosa. These biopsies were then distributed randomly among 3 weighboats, each containing 25 mls of appropriate media. For each experiment, 18 biopsies were used.

B. Incubation

After being washed with media the biopsies were placed in 25 x 100 culture test tubes (one biopsy/tube). The tubes each contained 1 ml of media and had been previously oxygenated for 45 minutes in a shaker bath at 37^{0}C. The tubes were now capped and the biopsies were incubated in the shaker bath (37^{0}C) for 2 hours. A pre-incubation period of 2 hours and transfer to new oxygenated media was used in some experiments. Three sets of 6 tubes (total of 18 tubes) were used in all experiments.

C. Assays

After the incubation period, pepsinogen assays were done on medium from each tube to determine the amount of secretion (see Appendix A).

Pepsinogen has no measurable proteolytic activity and therefore must first be converted to pepsin for assay[40]. While pepsin can act on a variety of substances and at a pH range of less than 1 - 5.5, its actions can be divided into two categories:

1. Limited proteolysis-- pH 3.5-5.5 Pepsin produces changes in physical properties (viscosity, clotting, solubility, etc.) but does not release breakdown products.

2. Extensive proteolysis-- pH 1.0-3.5 This can be measured quantitatively by the appearance of breakdown products, release of dye, or loss of solid substrate[71].

The most widely-used method is the digestion of denatured hemoglobin at pH 1.7-2.0[72]. As originally defined, 1 peptic unit is the activity of pepsin which releases 0.1 umole of tyrosine from 5.0 ml 2% hemoglobin (i.e. 100 mg) at pH 1.7 in 10 minutes at 37^{0}C.

In order to determine mgs protein/biopsy, Lowry assays were done on each tissue sample (see Appendix B).

D. Statistics

In each experiment two controls were used. On the basis of previous studies in this laboratory (D.E. Schafer, personal communication), pepsinogen secretion induced by 10^{-4}M ACh/eserine was considered to represent maximal secretion and therefore this was taken as the

positive control. Non-stimulated pepsinogen secretion occurring with T8 or K-R bicarbonate media alone was the negative control.

Pepsinogen secretion was first calculated in peptic units. One peptic unit is defined as the activity of pepsin which releases 0.1 umole tyrosine from 5.0 ml 2% hemoglobin at pH 1.7 in 10 minutes at 37^{0}C. On the basis of the previous work of other investigators in our laboratory, peptic units were obtained by multiplying the optical density of the sample (at 760) by the factor 47.8. The amount of protein in each sample was determined by Lowry assay, and peptic units/mg protein were then calculated.

Because the positive controls varied considerably between experiments, for comparison of results the mean secretion induced by positive controls was defined as 100% stimulation. Secretion by the negative controls and all other experimental groups was expressed as % of positive controls $\pm$ 1 standard error of the mean. Additionally, Student's t-tests were done to determine statistical significance (p).

Materials:

Trowell's 8 (T8)

Trowell's 8 with $SrCl_2$ (2 mM)

Trowell's 8 without $CaCl_2$

Trowell's 8 without $CaCl_2$, with $SrCl_2$

ACh/eserine T8. This medium was prepared by dissolving ACh chloride in (T8 with eserine). The final concentration of eserine was 0.2 mg/ml (7×10^{-4}M); final concentration of ACh was 0.02 mg/ml (10^{-4} M).

Krebs-Ringer bicarbonate solution with 100 mgs% glucose (K-R medium)

Krebs-Ringer bicarbonate solution with 100 mgs% glucose and with amino acids: MEM amino acids were added to the K-R medium in a concentration of 1:100.

Carbachol/K-R medium. Carbachol was dissolved in K-R medium. Final concentration was 0.02 mg/ml (10^{-4} M).

Veratridine. Veratridine was sonicated in T8 medium. A final concentration of 10^{-4} M was used.

X537A. Final concentration was 25 ug/ml.

A23187 (gift of Robert L. Hamill, Ph.D. of Eli Lilly). Stock solution was prepared by dissolving 10 mg A23187 in 1 ml DMSO (see below). Culture media contained either 1, 5, or 10 ug/ml A23187, with the DMSO concentration never exceeding 0.1%.

III. RESULTS

A. Pepsinogen Assay

The pepsinogen assay involves the activation of pepsinogen to pepsin and the peptic proteolysis of denatured hemoglobin. The assay is affected by:

1. concentration of enzyme (pepsinogen)
2. concentration of substrate (hemoglobin)
3. activation time (with HCl)
4. incubation time
5. pH
6. other ions in the medium

Experiments were undertaken to confirm the optimal conditions for this assay and to check the effects of any modifications.

Activation time: When the activation time with HCl was varied from 0 to 20 minutes, no significant change was noted in the extent of reaction (as measured by optical density of the reaction product). (# experiments = 2)

Incubation time: As the incubation time with hemoglobin was increased in 10 minute intervals from 0 to 60 minutes, the optical density was found to increase linearly. (# experiments = 3)

pH: Highest readings were obtained with 0.04 N HCl as opposed to 0.02 N or 0.08 N HCl. (# experiments = 1)

Media: When compared to distilled H_2O as the control, neither T8 nor K-R bicarbonate medium had any significant effects on the assay.

(# experiments = 1)

Other investigators in the laboratory had been using a pepsinogen assay involving a 10 minute activation with 0.04 N HCl and a 40 minute incubation period with hemoglobin. For comparison purposes it was decided not to alter these assay conditions (although the optical density of the reaction product is greater with a 60 minute incubation).

B. Concentration of extracellular Ca^{++}

To determine whether extracellular Ca^{++} is essential for stimulation of secretion, the effect of Ca^{++}- free medium was investigated. In these experiments biopsies were incubated for 2 hours with either control T8, ACh/eserine T8, or Ca^{++}- free ACh/eserine T8. No significant depression of secretion was noted with the omission of Ca^{++}. However, 0.01-0.19 mg% Ca^{++} was detected in the "Ca^{++}- free" media after the 2 hour incubation. (4 samples were sent to the V.A. Clinical lab for Ca^{++} analysis).

Because significant depression of secretion with Ca^{++}- free media has been reported in numerous other systems[2-5] and since Ca^{++} was detected in the "Ca^{++}- free" media after the 2 hour incubation, it was decided to alter the experimental procedure. A 2 hour pre-incubation period was used in subsequent experiments in order to decrease the extracellular Ca^{++} level and stabilize the tissue biopsies before exposing them to ACh stimulation (see figure 2).

With this pre-incubation procedure, the omission of Ca^{++} from the ACh/eserine T8 medium depressed mean pepsinogen secretion to 62 $\pm$ 6% of the positive control. This depression was statistically significant in

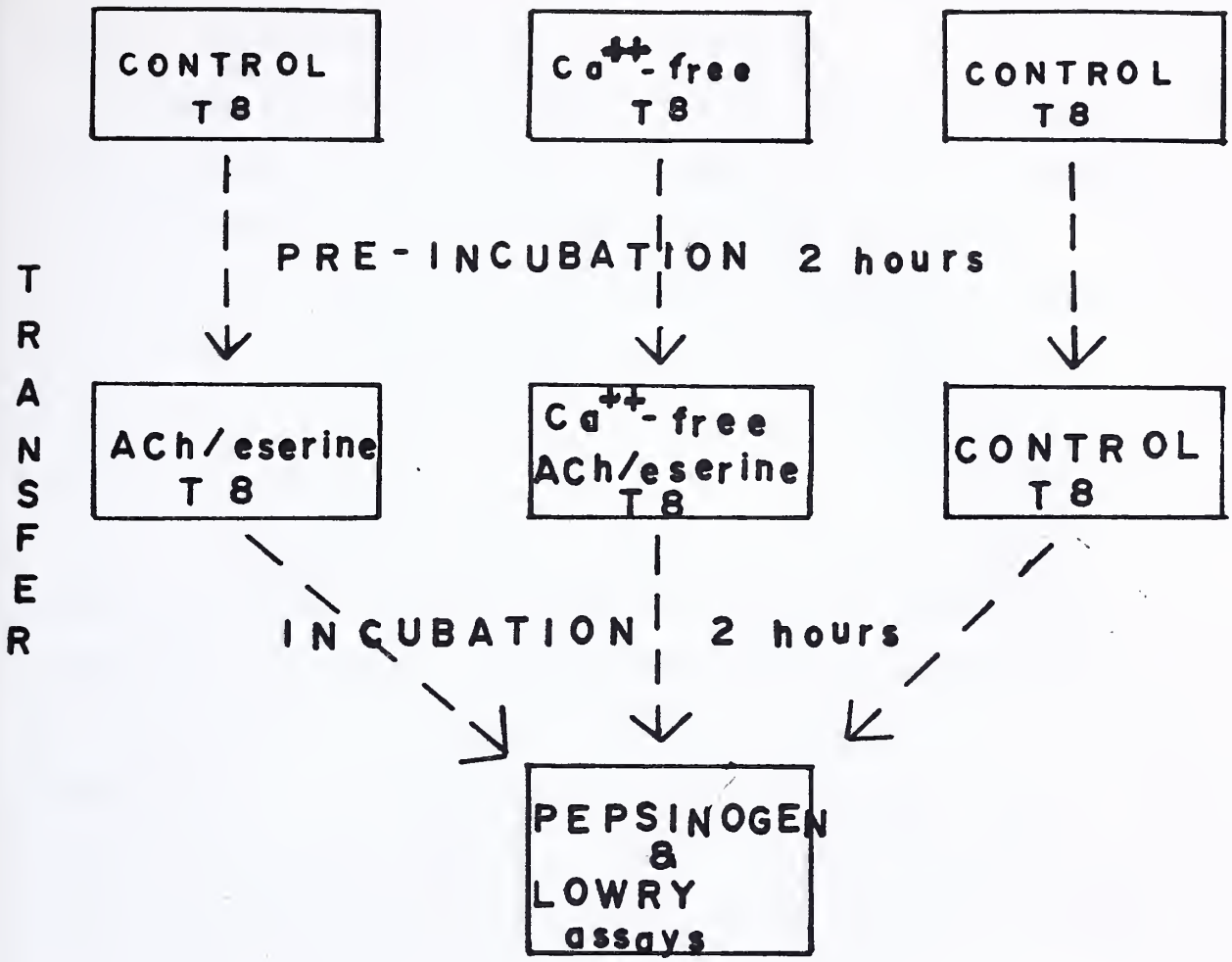


FIGURE 2. EXPERIMENTAL PROCEDURE: EFFECT OF Ca^{++} DEPRIVATION ON PEPSINOGEN SECRETION

2 of the 5 experiments ($p < 0.05$). (See figure 3.)

Pepsinogen secretion by the positive control (ACh/eserine T8) increased with pre-incubation from 17 $\pm$ 2 peptic units to 43 $\pm$ 8 peptic units ($p < 0.001$). Also, control levels were lower. Pepsinogen secretion by non-stimulated tissue was reduced from a mean of 32 $\pm$ 3 % (no pre-incubation) to 14 $\pm$ 2% (with pre-incubation). This was significant at $p < 0.001$. No Ca^{++} was detected in the media after the 2 hour incubation.

Thus it was demonstrated that reduction of extracellular Ca^{++} depressed pepsinogen secretion. Next, the effect of increasing Ca^{++} concentrations was studied. Significant problems with precipitation were encountered in preparing a T8 medium with higher Ca^{++} concentrations. T8 is a complex medium containing inorganic salts, glucose, insulin, amino acids, and vitamins. In order to determine the ability of gastric mucosa to secrete pepsinogen in the absence of amino acids, and to more easily manipulate the Ca^{++} concentration, a simpler medium was used: Krebs-Ringer bicarbonate solution with 100 mg% glucose.

The following results were obtained:

1. Pepsinogen secretion with ACh/eserine K-R bicarbonate medium was significantly less than that induced by ACh/eserine T8 (19 $\pm$ 5 peptic units compared with 43 $\pm$ 8 peptic units; $p < 0.05$).

2. Addition of amino acids to the K-R medium resulted in a depression of pepsinogen secretion from 52 $\pm$ 12 peptic units to 28 $\pm$ 6 peptic units ($p < 0.05$).

3. No significant change in pepsinogen secretion occurred with an

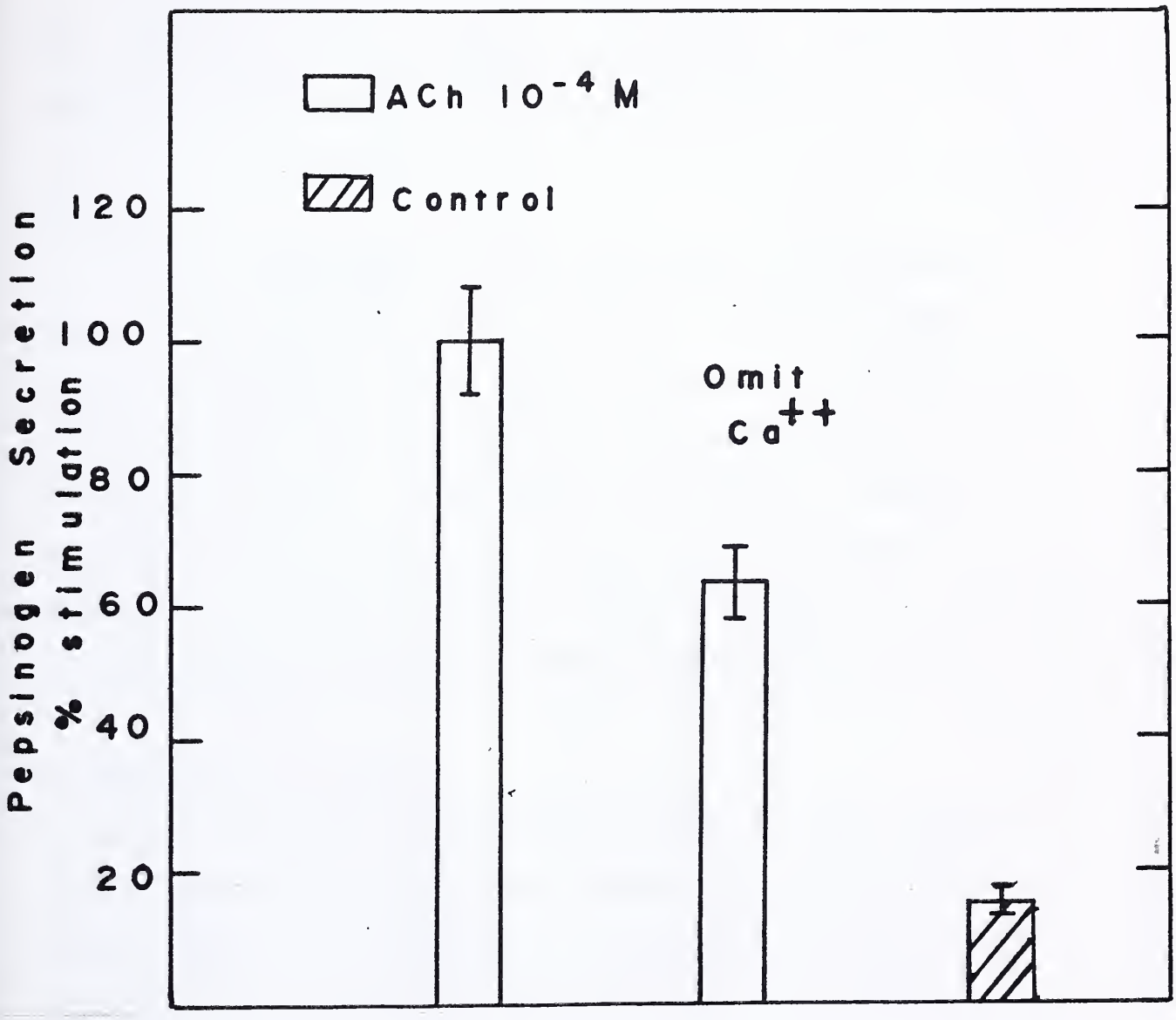


FIGURE 3. EFFECT OF Ca^{++} DEPRIVATION ON PEPSINOGEN SECRETION (# experiments = 5)

Gastric biopsies pre-incubated in T8 were transferred after 2 hours to either T8 (Control) or T8 with ACh/eserine. Biopsies pre-incubated in Ca^{++}- free T8 were transferred after 2 hours to Ca^{++}- free ACh/eserine T8.

extracellular Ca^{++} concentration of 5.0 mM or 7.5 mM. (See figure 4.)

4. A 10 mM concentration of Ca^{++} depressed secretion from 18 $\pm$ 7 peptic units to 5 $\pm$ 1 peptic units ($p < 0.05$). However, precipitation was observed in the medium. (See figure 4.)

C. Effect of Sr^{++}

It has been demonstrated in other systems that Sr^{++} is capable of substituting for Ca^{++} in the stimulus-secretion process[34]. In this system, no significant change in secretion occurred when Sr^{++} was added to the Ca^{++}- containing ACh/eserine T8 medium. However, addition of Sr^{++} to Ca^{++}- free ACh/eserine T8 resulted in a marked inhibition of mean secretion to 51 $\pm$ 6 % of the positive control. Depression was significant ($p < 0.05$) in 3 of the 5 experiments. No pre-incubation period was used in these experiments. (See table 1)

D. ACh/eserine vs. Carbachol

Because acetylcholinesterase (AChE) is present in tissue, ACh is rapidly inactivated. For this reason, eserine (physostigmine) sulfate, a potent and specific AChE inhibitor, was added to the ACh/T8 medium (and K-R bicarbonate medium) in a concentration of 7×10^{-4} M/liter (0.2 mg/ml).

To determine whether sufficient eserine was available to inhibit AChE, secretion induced by the ACh/eserine medium was compared to that stimulated by carbachol. Carbachol is the carbamyl ester of choline and, unlike ACh, is not susceptible to cholinesterase.

It was found that carbachol induced a secretion of 55 $\pm$ 12% when

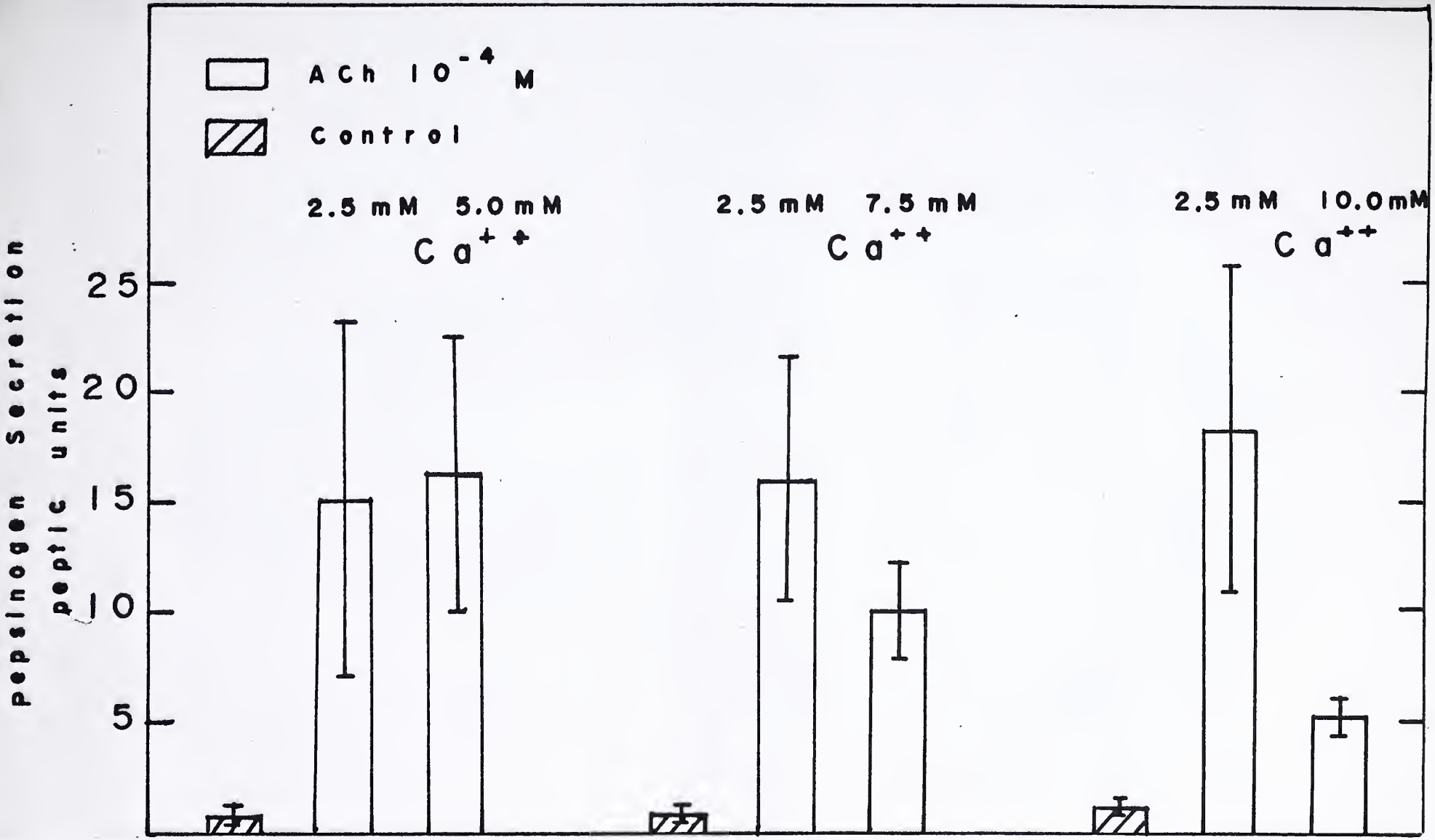


FIGURE 4. EFFECT OF EXTRACELLULAR Ca++ CONCENTRATION ON PEPSINOGEN SECRETION
(# experiments = 3) Gastric mucosa biopsies were pre-incubated in control K-R bicarbonate media with 2.5 mM Ca++. After 2 hours biopsies were transferred to either control K-R bicarbonate, K-R bicarbonate with ACh/eserine, or K-R bicarbonate with ACh/eserine and higher Ca++ concentrations (5.0, 7.5, 10.0 mM). Pepsinogen secretion is expressed in peptic units.

	Ca^{++}	Omit Ca^{++}
Omit Sr^{++}	100 ± 10%	84 ± 10%
Sr^{++}	90 ± 8%	51 ± 6%

TABLE 1. EFFECT OF Sr^{++} ON PEPSINOGEN SECRETION (# experiments = 12) Gastric biopsies were incubated 2 hours with 10^{-4} M ACh/eserine in T8 along with various combinations of Ca^{++} (2.0 mM) and Sr^{++} (2.0 mM) (as depicted). Biopsies incubated 2 hours in T8 with Ca^{++} but without ACh/eserine served as the negative controls (not shown in table).

compared with ACh/eserine ($p < 0.05$) (# experiments = 2). This experiment was not repeated with increasing concentrations of carbachol. Reasons for continuing to use ACh/eserine rather than carbachol in our experiments included:

1. ACh is a more potent secretogogue than carbachol in this system.

2. The physiological stimulus for pepsinogen secretion is ACh rather than carbachol.

3. When compared with ACh, carbachol has additional nonspecific pharmacological properties[73].

E. Veratridine

Veratridine has been shown[67] to depolarize membranes, thereby increasing membrane permeability to Ca^{++}. To study this effect, gastric mucosa was incubated 2 hours with 10^{-4} M veratridine in the presence of Ca^{++}. A mean stimulation of pepsinogen secretion to 52 $\pm$ 3% was demonstrated with veratridine. In five of six experiments, secretion was significant ($p < 0.05$). (See figure 5.)

F. X537A

Biopsies were incubated for 2 hours with X537A (a non-specific Ca^{++} ionophore) to assess the effect of increased intracellular Ca^{++} concentration on secretion. X537A was used in a concentration of 25 ug/ml, as described in the method of Nakazato & Douglas[66]. In the presence of Ca^{++}, X537A induced a mean pepsinogen secretion of 49 $\pm$ 4% of the positive control. The stimulation by X537A in each of the 3 experiments was significant at $p < 0.001$. (See figure 6.)

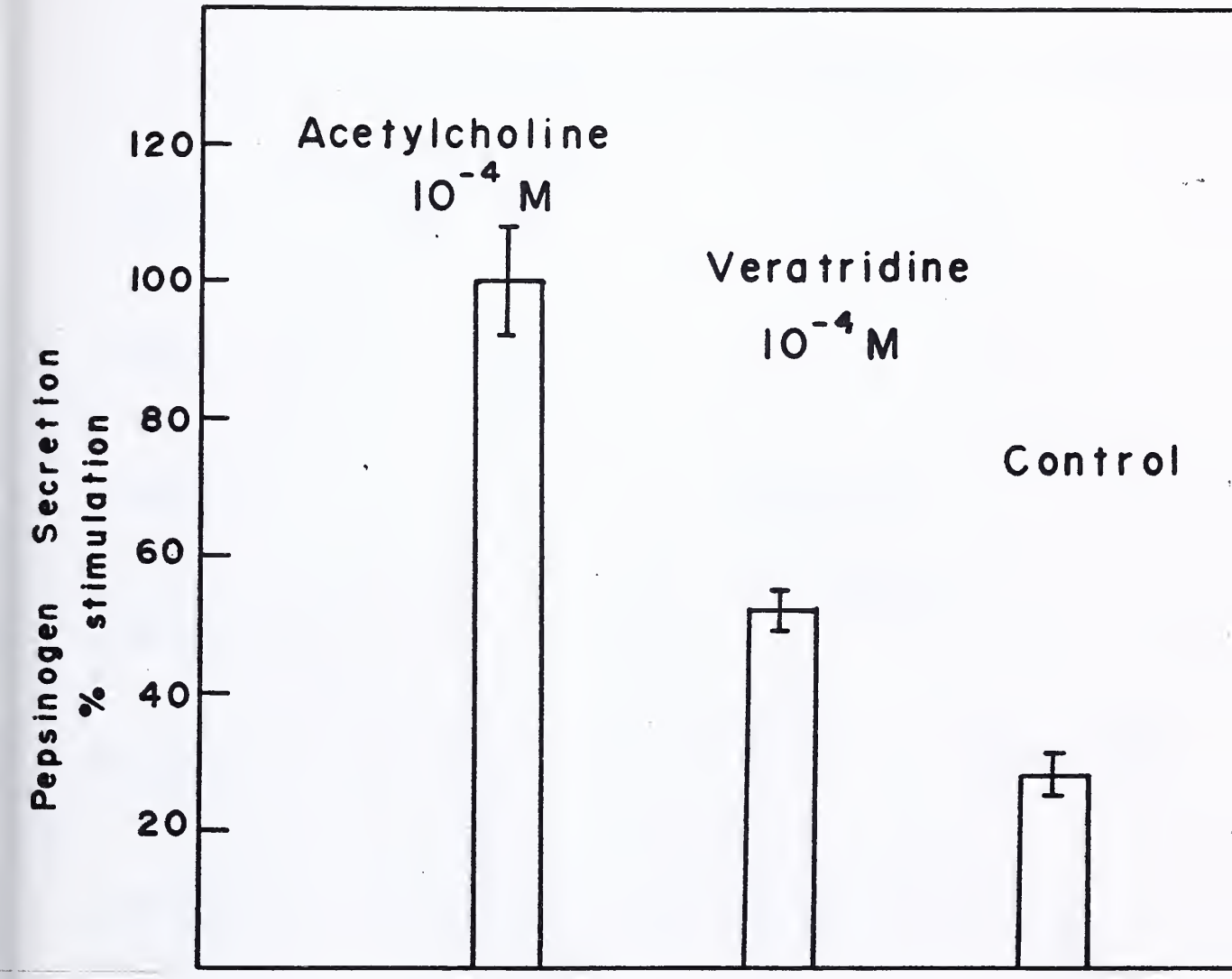


FIGURE 5. EFFECT OF VERATRIDINE ON PEPSINOGEN SECRETION (# experiments = 6) Gastric mucosa biopsies were incubated for 2 hours in T8 medium with either ACh/eserine (positive control), veratridine, or with T8 (negative control).

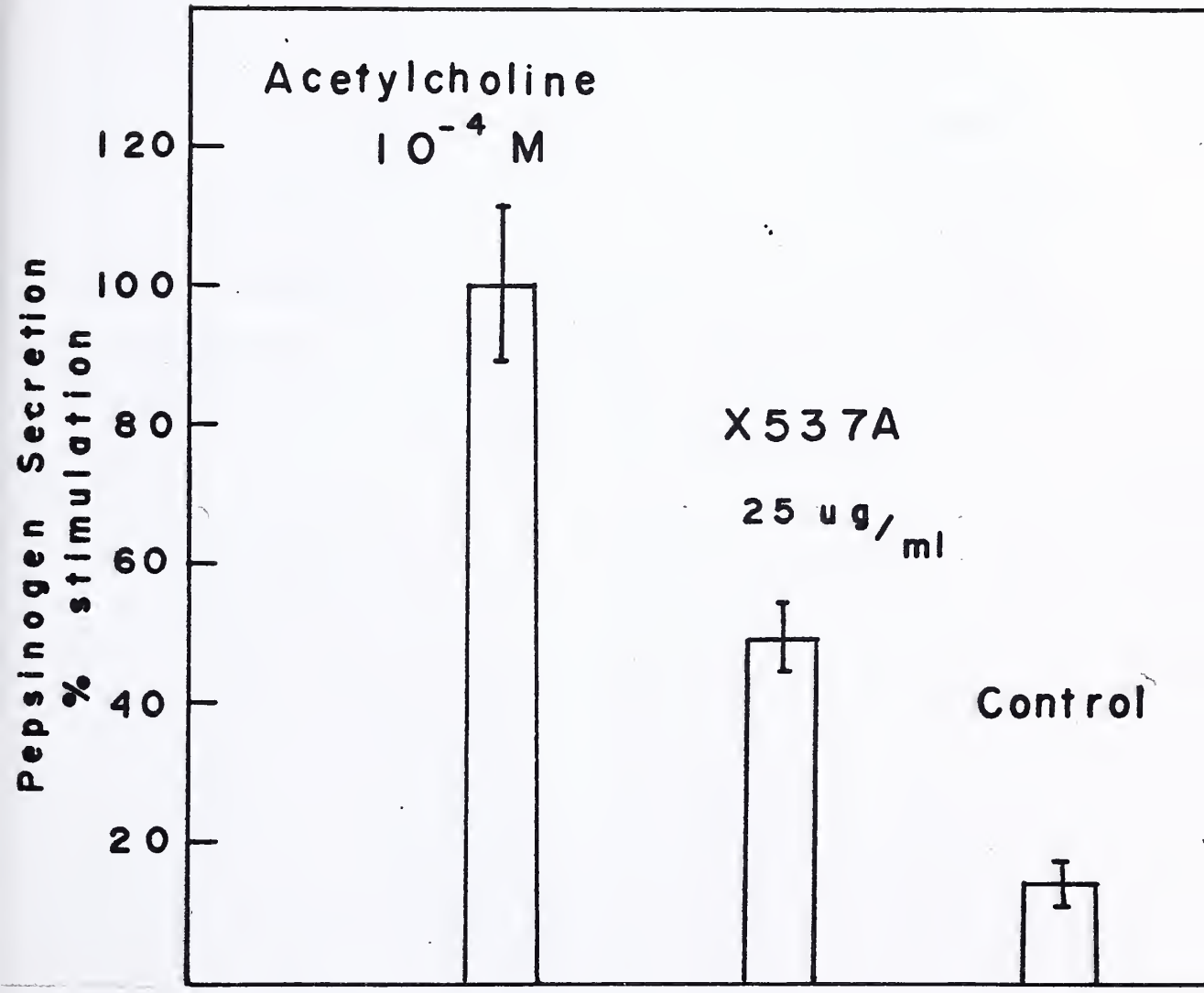


FIGURE 6. EFFECT OF X537A ON PEPSINOGEN SECRETION (# experiments = 3) Gastric mucosa biopsies were incubated 2 hours in T8 medium with either ACh/eserine (positive control), X537A, or with T8 alone (negative control).

G. A23187

A23187, a more specific Ca^{++} ionophore, was solubilized in dimethyl sulfoxide (DMSO), with the final DMSO concentration not exceeding 0.1%. This concentration of DMSO has been shown to have no effect on the morphology or secretory response of mast cells[64]. Tissue biopsies were then pre-incubated for 2 hours with A23187 in Ca^{++}- free medium according to the method of other investigators[65]. After pre-incubation, the biopsies were transferred to media containing A23187 and Ca^{++} for a 2 hour incubation. No significant amount of secretion was observed with 1 ug/ml, 5 ug/ml, or 10 ug/ml of A23187. In one experiment, a pre-incubation time of 15 minutes was used; again no significant secretion was noted. (# experiments = 5)

IV. DISCUSSION

A. Extracellular Ca^{++} and secretion

In the experiments without a pre-incubation period, no significant depression of pepsinogen secretion in response to ACh over 2 hours was demonstrated when Ca^{++} was omitted from the medium. When Ca^{++} was detected in the Ca^{++}- free medium after the 2 hour incubation period, we attempted to reduce the extracellular Ca^{++} concentration further. It was decided not to use EDTA or EGTA to chelate the excess Ca^{++} as these have been shown to alter membrane permeability in various tissues[74]. Instead, the tissue biopsies were pre-incubated for 2 hours in normal and Ca^{++}- free media before testing the effect of 10^{-4}M ACh. With this procedure, mean pepsinogen secretion in response to ACh without Ca^{++} was depressed to 62 $\pm$ 6% of the response obtained with ACh and Ca^{++} (# experiments = 5). The depression was significant at p 0.05 in 2 of the 5 experiments. Thus, extracellular Ca^{++} was shown to be influential but not essential for secretion in this system.

Schafer & Donaldson[75] studied protein secretion and synthesis in rabbit gastric mucosal suction biopsies maintained in organ culture. They found that omission of Ca^{++} significantly depressed protein secretion but not synthesis. With the addition of 1 mM EGTA, however, secretion was totally inhibited while synthesis was depressed by one-third. It was then proposed that moderate Ca^{++} depletion affects secretion and more severe depletion also affects protein synthesis.

In the adrenal medulla, Douglas & Rubin[76] reported that omission

of extracellular Ca^{++}, without addition of any chelating agent resulted in a marked depression and even abolishment of secretion . Re-introducing Ca^{++} restored the secretory response to ACh. Such a phenomenon has been demonstrated in other tissues such as the salivary gland[23], neurohypophysis[21], spleen[77], and exocrine pancreas[79,80]. However, Case & Clausen[78] report that the rate of amylase release from rat pancreas was not affected by extracellular Ca^{++} levels from 0.1 - 2.5 mM, and it was concluded that extracellular Ca^{++} is not of great importance for secretion. Similarly, carbachol-induced secretion in rabbit pancreas was found to occur in a Ca^{++}-free medium containing EGTA[81].

Pepsinogen secretion was not abolished by omission of Ca^{++} in our experiments, and also it was not enhanced by increased extracellular Ca^{++} levels. Schafer & Donaldson[75] found no change in pepsinogen secretion with a Ca^{++} concentration of 4 mM. However, others have shown that the secretory response varies directly with the Ca^{++} concentration[77]. Hales & Milner[82] report that insulin secretion varied directly with Ca^{++} concentrations up to 2 - 3 mM. With higher levels the response was constant, while above 10 mM it was diminished. In our system depression of mean pepsinogen secretion was observed with an extracellular Ca^{++} level of 10 mM; this was not statistically significant (p 0.05).

It thus seems that in our experimental model, extracellular Ca^{++} is not rate-determining in pepsinogen secretion but is most likely important for maximal secretion to occur (perhaps by influencing the plasma membrane). This is not incompatible with the stimulus-secretion

hypothesis. The increase in cytoplasmic Ca^{++} concentration hypothesized to occur after stimulation may originate from intracellular stores rather than from the extracellular medium. It is known that several cellular structures actively transport or store Ca^{++} and maintain the free intracellular Ca^{++} concentration at low levels (10^{-5} - 10^{-7} M, measured directly by Baker[83]). These components include the plasma membrane[84], sarcoplasmic reticulum[85], endoplasmic reticulum[86], and mitochondria[87]. The increase in cytoplasmic Ca^{++} concentration hypothesized to occur after stimulation may originate from intracellular stores rather than from the extracellular medium. This idea has recently been supported by various experimental methods. Using the fluorescent probe chlorotetracycline (CTC) which complexes to Ca^{++}, Chandler and Williams[60] demonstrated release of Ca^{++} from intracellular membranes of pancreatic acinar cells during bethanechol stimulation. Another method involves studying the kinetics of labelled Ca^{++} ($^{45}Ca^{++}$) in cells during stimulation. It was found that ACh caused efflux of $^{45}Ca^{++}$ from cat submandibular gland. Cholecystokinin (CCK) and ACh also accelerated the release of $^{45}Ca^{++}$ and amylase from rat pancreas, independent of the extracellular Ca^{++} concentration from 0.1 - 2.5 mM[78]. In this study, secretion occurred only if the cell membrane had not been previously depleted of Ca^{++} by EGTA.

It is possible, therefore, that pepsinogen secretion involves mainly the release of Ca^{++} from intracellular stores, and not entry of Ca^{++} from the extracellular medium. The fact that significant

depression of pepsinogen secretion occurred only with prolonged exposure of the tissue to Ca^{++}- free medium supports this idea.

Alternatively, Ca^{++} may not be the direct mediator of pepsinogen secretion but instead may act at another site in the secretory process. It is possible that cyclic nucleotides, for example, are affected by intracellular Ca^{++} levels and that the nucleotides then induce secretion. However, the role of cyclic nucleotides in pepsinogen secretion has not been fully investigated. Previous experiments by Schafer & Donaldson[75] have failed to show stimulation of secretion with dibutyryl cyclic GMP.

B. Veratridine, X537A, and A23187

By raising intracellular Ca^{++} levels, certain non-physiological stimuli can induce secretion. This is indirect evidence that Ca^{++} is a coupling factor in the normal physiological stimulus-secretion process. In the present study, three drugs were used to assess the effect of increased intracellular Ca^{++}: veratridine, X537A, and A23187. Veratridine activates the action potential Na^{+} ionophore, thereby depolarizing the cell membrane and increasing Ca^{++} permeability. At a concentration of 10^{-4}M veratridine did induce a significant pepsinogen secretion in 5 of the 6 experiments (p 0.05). X537A, a non-specific Ca^{++} ionophore, also induced pepsinogen secretion of equal magnitude (significant in all 3 experiments at p 0.001). However, A23187 (a specific Ca^{++} ionophore) did not cause any significant secretion. This is similar to findings reported by Nakazato & Douglas[66]: X537A, but not A23187, caused release of

vasopressin from rat neurohypophysis. Also, Schafer & Donaldson[75] could not demonstrate pepsinogen secretion from rabbit gastric mucosa in organ culture using A23187. X537A is able to transport monovalent cations while A23187 is not. It is possible that X537A, like veratridine, causes a Na^+ influx which leads to depolarization. Supporting this view, Ito et al found that X537A-induced noradrenaline secretion increased with increasing concentrations of external Na^+ [90].

Thus, while A23187 is the more specific Ca^{++} ionophore it did not induce pepsinogen secretion in this system. Such a result has been reported in various other tissues (e.g. adrenal medulla, posterior pituitary), especially if the tissues were exposed to A23187 in the presence of Ca^{++}. However, if tissues are incubated with A23187 in the absence of Ca^{++}, secretion may occur upon re-introduction of Ca^{++} [58,90]. Chandler[58] has shown that the rate of A23187 uptake is reduced up to 20-fold in the presence of Ca^{++} and Mg^{++}. Nevertheless, pepsinogen secretion could not be induced in our system despite pre-incubation in a Ca^{++}- free and Mg^{++}- free medium.

Another hypothesis suggests that the Ca^{++} ionophores release intracellular Ca^{++} stores rather than cause an influx of extracellular Ca^{++}. A23187, for example, has been shown to induce Ca^{++} release from isolated mitochondria and isolated sarcoplasmic reticulum[56]. Also, the Ca^{++} ionophores have been shown in certain tissues to increase the concentration of cytosolic Ca^{++} even in the absence of extracellular Ca^{++} [91]. Whatever the mechanism involved (i.e. Na^+ influx, transport of extracellular Ca^{++}, or Ca^{++} release from intracellular stores) it is apparent that both X537A and

A23187 have been shown to induce secretion. While A23187 is the more specific Ca^{++} ionophore, the effect of X537A is more reliable.

C. Basal secretion of pepsinogen

Non-stimulated pepsinogen secretion (controls) in those experiments with pre-incubation averaged 14 ± 2% of secretion by ACh-stimulated tissue. In comparison, in experiments without pre-incubation, controls averaged 32 ± 3%. However, in absolute peptic units there was no significant difference between the two control groups.

Pepsinogen secretion by unstimulated tissue might represent damage to the cells with subsequent non-physiologic release of pepsinogen. If this were the case one might expect pre-incubation to stabilize the cells, thereby decreasing pepsinogen release. In fact, pre-incubation did not significantly affect secretion expressed in peptic units. It is possible, therefore, that secretion in the control group represents more than traumatic release of pepsinogen. In man, for example, unstimulated pepsinogen secretion is 28% of that induced by histamine stimulation[88]. Also, *in vivo* studies of herbivores have demonstrated continuous basal secretion[89].

D. Strontium and pepsinogen secretion

In 1911 Mines found that neuromuscular transmission could occur in a Ca^{++}- free medium with the addition of either Sr^{++} or Ba^{++} but *not* Mg^{++}. It seemed unlikely, therefore, that the divalent nature of Sr^{++} and Ba^{++} could account for their ability to substitute for Ca^{++}. Mines postulated that the effective substitute cations are

able to combine chemically with certain tissue sites, while Mg^{++} could not[93].

Since 1911, Sr^{++} has been found to substitute for Ca^{++} in various secretory systems[34,35]. In the adrenal medulla, for example, when added with ACh or excess K^{+}, Sr^{++} induces catecholamine secretion. Also, Sr^{++} has been shown to be more effective than Ca^{++} in effecting histamine release from mast cells[36].

The effect of Sr^{++} on pepsinogen secretion was studied during the early part of this investigation and therefore no pre-incubation period was used. It was shown that addition of Sr^{++} to Ca^{++}- containing ACh medium had no appreciable effect. This is consistent with the finding that excess Ca^{++} (5.0 - 10.0 mM) also did not enhance pepsinogen secretion. In contrast, Douglas & Rubin[34] reported that both excess Ca^{++} and excess Sr^{++} increase secretion from the adrenal medulla.

In these experiments (in which no pre-incubation was used) omission of Ca^{++} did not result in depression of secretion. However, addition of Sr^{++} to the Ca^{++}- free ACh medium resulted in depression of mean secretion (51 $\pm$ 6%), significant in 3 of the 5 experiments (p 0.05). One immediately apparent explanation is that Sr^{++} cannot substitute for Ca^{++} and indeed is even inhibitory. However, this would be unusual in view of the ability of Sr^{++} to induce secretion in so many other systems.

Alternatively, another explanation involves the assumption that sufficient Ca^{++} was present in the Ca^{++}- free medium for secretion to occur. No pre-incubation period was used and it is possible that

the tissues were not depleted of Ca^{++} by the 2 hour incubation with Ca^{++}- free medium. This is supported by the actual detection of Ca^{++} (up to 0.19 mg%) in the Ca^{++}- free media after incubation.

The presence of sufficient Ca^{++} would explain why no significant depression of secretion occurred with the omission of Ca^{++} from the ACh media. When Sr^{++} was added to the Ca^{++}- free ACh media, however, depression did occur. Nevertheless, secretion was still considerably higher than control levels. It is possible, therefore, that Sr^{++} is indeed able to activate the pepsinogen secretory process, but that it is a less effective cation in this respect than Ca^{++}. Under conditions in which more Sr^{++} than Ca^{++} is present in the medium, one may assume that the critical sites involved in secretion will be presented with Sr^{++}. If Sr^{++} is less effective than Ca^{++}, as postulated, then sub-maximal secretion will occur. Indeed, similar experiments on neurotransmitter release have shown that while Sr^{++} is able to substitute for Ca^{++}, it is less effective: secretion with 2.0 mM Sr^{++} was equivalent to that induced with 0.3 mM Ca^{++} [35].

VI. APPENDICES

APPENDIX A. Pepsinogen Assay

Reagents: 0.04 N HCl
2% Hemoglobin (Hb) in 0.03 N HCl
Folin's Reagent
5% TCA
0.2N NaOH

Method:

a. After the sample was centrifuged for 10 minutes x 8000 RPM, 0.1 ml of the supernatant was placed in a 16 x 100 test tube (in duplicate).

b. 0.03 ml of 0.04 N HCl was then added to each sample; all samples were placed in a shaker bath at 37^{0}C.

c. After an activation time of 10 minutes, 0.8 ml of 2% Hb was added and the samples were incubated further for 40 minutes at 37^{0}C.

d. 1.8 ml of 5% TCA were then added and the samples were removed from the incubator.

e. After 30 minutes the samples were filtered (grade 4 filter paper).

Colorimetry:

a. To 1 ml of the filtate, 5 ml of 0.2 N NaOH were added.

b. Samples were activated for 10 minutes, and then 0.5 ml Folin's reagent was added.

c. After 30 minutes, the samples were read using a spectrophotometer at 760.

APPENDIX B. LOWRY ASSAY

Reagents:

A 2% Na_2CO_3 in 0.1 N NaOH

B mix B1 and B2 (1:1)

B1 1% $CuSO_4 \cdot 5\ H_2O$

B2 NaK tartrate (2%)

C 50 ml reagent A and 1 ml reagent B

E Folin-Ciocalteau phenol reagent - 1 N

Method:

a. Test solutions should not exceed a maximum of 0.5 mg total protein. Test solutions were prepared by grinding biopsies in 1.5 ml of 0.5% Na desoxycholate.

b. Dilute 0.3 ml of test solutions to 1 ml with H_2O.

c. Add 5 ml reagent C. Vortex immediately.

d. Wait 30 minutes and read samples in a spectrophotometer at 780.

APPENDIX C. TABLE 1. Trowell 8 Medium (Gibco)

Component:	mg/L
Inorganic Salts	
$CaCl_2$ (anhydrous)	220.0
KCl	450.0
$MgSO_4 7H_2O$	250.0
NaCl	6100.0
$NaHCO_3$	2820.0
$NaH_2PO_4H_2O$	398.0
Glucose	4000.0
Insulin (bovine)	50.0
Phenol red	10.0
Amino Acids	
L-arginine HCl	21.0
L-cysteine HCl	47.0
L-histidine HCl	10.0
L-isoleucine	26.0
L-leucine	26.0
L-lysine	36.0
DL-methionine	15.0
L-phenylalanine	33.0
L-threonine	48.0
L-tryptophane	4.0
L-tyrosine	18.0
L-valine	23.0
Vitamins	
Para-aminobenzoic acid	35.0
Thiamine HCl	17.0

APPENDIX D. TABLE 2. K-R Bicarbonate Solution

Component:	mg/L
Inorganic Salts	
$CaCl_2$ (anhydrous)	282.0
KCl	355.0
$MgSO_4$ $7H_2O$	294.0
NaCl	6935.0
$NaHCO_3$	2080.0
KH_2PO_4	162.0
Glucose	1000.0

APPENDIX E. TABLE 3.

MEM Amino acids without glutamine (50X)			T8 Medium
Amino Acid	mg/L		mg/L
L-arginine · HCl	6320	(300X T8)	21
L-cystine	1200		---
L-histidine HCl· H_2O	2100		---
L-isoleucine	2625	(100X T8)	26
L-leucine	2620	(100X T8)	26
L-lysine HCl	3625	(100X T8)	36
L-methionine	755	(50X T8)	15
L-phenylalanine	1650	(50X T8)	33
L-threonine	2380	(49X T8)	43
L-tryptophane	510	(127X T8)	4
L-tyrosine	1800	(100X T8)	18
L-valine	2340	(100X T8)	23
L-cysteine	---		47
L-histidine HCl	---		10

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